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Gene expression in precursor cells of prostate cancer associated with activin by combination of subtractive hybridization and microarray technologies

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Received 12 November 2003

Abstract

Prostatic intraepithelial neoplasia (PIN) is considered the pre-malignant stage of prostate carcinoma, but little is known of its initiation and evolution. The identification of genes associated with these precursors of prostate cancer may elucidate the pathways of the early oncogenesis of this disease. Previously, we have reported that activin, a member of the TGF β superfamily, acted as an inhibitory growth factor in prostate cancer. We used laser capture microdissection, mRNA-library amplification (RNA-PCR), subtractive hybridization, and complementary DNA microarray to examine gene expression profiles in activin-positive PIN, compared with activin-negative PIN. Subtractive hybridization showed that 28 genes were differentially expressed (13 and 15 genes were up- and down-regulated, respectively). Microarray analysis identified 29 and 56 more genes (4 times) up- and down-regulated, respectively, suggesting that DNA microarray is a more effective method in screening gene profiles. We have validated the known genes identified by both subtractive hybridization and microarray technologies, using Northern blot analysis in the mRNA libraries generated from cells microdissected from pathological slides. We have successfully showed that at least 13 genes are involved in activin-associated PIN. The evaluation of candidate genes that emerge from these experiments provides a rational approach to investigate those genes significant in evolution from PIN to prostate carcinoma.

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Keywords: Prostate cancer; Laser capture dissection; RNA-PCR; Microarray; PIN; hPSE; p53BP; Apoptosin; St14; ptc-like receptor

Prostate cancer accounts for 20% of all male malignancies and 11% of cancer deaths in men in the United States. There is no cure for advanced prostate cancer. The pathogenesis of converting prostatic intraepithelial neoplasia (PIN) to invasive carcinoma remains obscure. The identification of cell-specific, stage-specific genes in PIN may elucidate the pathways of the initiation and progression of this disease. PIN was originally described by McNeal [1] as an abnormal epithelial proliferation in the prostatic ducts, ductules, and acini, which is widely accepted as a pre-malignant condition and the strong association between PIN and prostate cancer has been reported [2,3].

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Previously, we and others have reported that activin, a member of TGF β family, acted as an inhibitory growth factor in prostate cancer in vitro and in vivo, mediating through inhibition of cell proliferation and enhancement of apoptosis [4–8]. In addition to growth changes and apoptosis, activin induces morphological changes in prostate cancer cells, which is accompanied by up-regulation of prostatic markers such as prostatic-specific antigen (PSA), prostatic acid phosphatase (PAP), and androgen receptor [9], presumably representing further cell differentiation. Using a novel subtractive hybridization method, we were able to identify p53 and p16 genes, which were associated with cell growth and apoptosis in activin-mediated LNCaP cells [10] and human prostate epithelial cells obtained from pathological slides [11], presumably representing further differentiation of these cells. Subsequently, we have

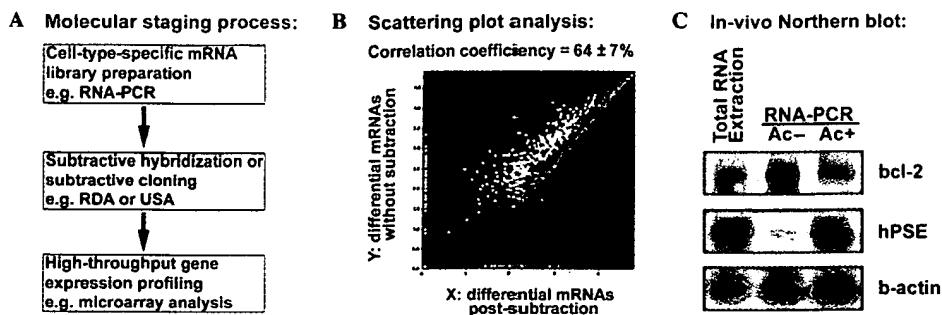


Fig. 1. Strategy for in vivo cancer staging using the procedure of RNA-PCR, subtractive cloning, and microarray analysis. (A) Three steps were comprised; first, single-cell RNAs were laser-microdissected and amplified by RNA-PCR; second, subtractive hybridization was performed to minimize the homologous background; and last, microarray analysis was applied to identify the entity of each individual differential gene. (B) Scattering plot comparison between differential gene results with and without subtraction hybridization showed a ~36% degree of differences in displayed gene populations, resulting from the deviation of high G-C gene contents and noisy heterogeneity backgrounds before subtraction. The subtractive hybridization was able to efficiently remove most of high deviation genes (white) and thus preserved the highly compatible representative genes (gray) for further microarray analysis. (C) The heterogeneity background from bulk tissues can be resolved using RNA-PCR-derived RNAs, which provide much higher resolution and sensitivity compared to phenol-chloroform extracted RNAs. The extracted total RNAs from one section slide of PIN tissues failed to distinguish the differential expressions of *bcl-2* and *hPSE* genes in activin-negative carcinoma cells in vivo, while the RNA-PCR-derived single-cell RNAs successfully identify the expressive differences as previously reported and as displayed by following microarray analysis.

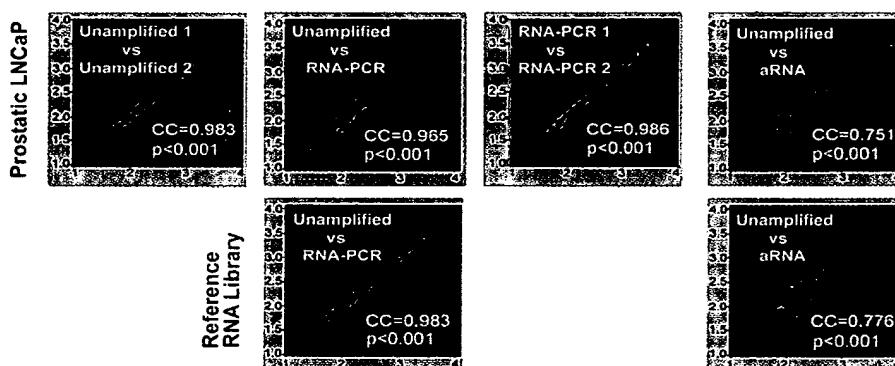


Fig. 2. Analysis of the representation rates among different RNA libraries isolated by phenol-chloroform extraction, RNA-PCR, and aRNA amplification. The comparison between RNA libraries of the same isolation methods showed a very high compatibility and consistency (>98% correlation coefficient). After two-cycle amplification, the RNA-PCR-derived poly(A)⁺ RNA library amplified from 50 LNCaP cells showed a ~96% correlation coefficient (CC) rate in comparison to the phenol-chloroform extracted total RNAs from about one million LNCaP cells. In the same fashion, the aRNA-amplification-derived RNAs from 50 LNCaP cells only provide a low ~75% CC rate. The same analysis procedure on a referenced RNA library instead of LNCaP cells showed similar results, indicating the consistency and reliability of using the RNA-PCR-derived poly(A)⁺ RNA library for single-cell microarray analysis in vivo.

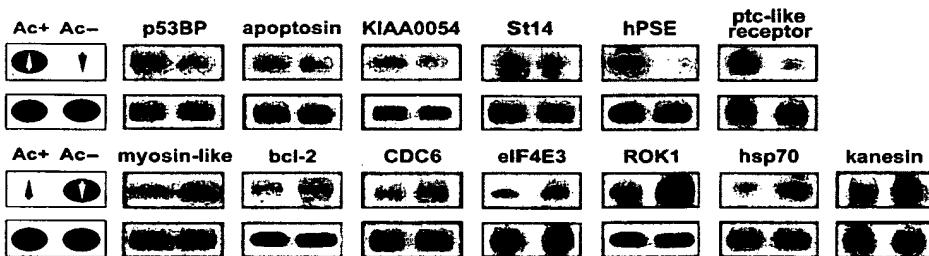


Fig. 3. Northern blot confirmation of microarray-identified differential genes between activin-positive (Ac+) and -negative (Ac-) PIN cells. About 60% identified differential genes (8/13) were found to be identical to the previously reported known genes obtained by subtractive hybridization and PCR. The other 40% newly found genes (5/13) were not detected by the subtractive hybridization and PCR protocol, probably due to the low resolution of gel electrophoresis and high noisy background of PCR amplification. The direct conjunction of subtractive hybridization and microarray analysis thus offers not only a convenient tool for identifying known differentially expressed genes but also a quick shortcut for screening unknown differential genes listed in the GenBank without known functions.

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Among the newly identified differentially expressed genes, we observed that St14, hPSE, and ptc-like receptor were up-regulated and CDC6, ROK1, and hsp70 down-regulated in activin-positive PIN cells, respectively. Suppression of tumorigenicity 14, St14, has been identified as one of the genes showing 5Aza-dC-induced aberrant methylation in pancreatic carcinoma [22], causing the reduction of cancer development. To the best of our knowledge, this is the first observation that St14 is associated with activin-associated PIN cells in prostate cancer. Patched (ptc), a multiple membrane spanning receptor TRC8 (TRC8), appears to participate in the development of kidney cancer [23,24] and may be implicated in hereditary renal carcinoma [25]. The role ptc-like receptor plays in the ptc-signaling pathway remains to be determined. Human prostate-specific Ets, a member of the Ets family, regulates the proliferation, differentiation, and development of prostate epithelial cells. Interestingly, this gene was also identified as one of the gene markers for low-grade prostate cancer [26]. Furthermore, negative immunoreactivity for hPSE strongly suggests malignancy in the prostate glands and decreased immunoreactivities of prostate glands for hPSE could suggest prostate carcinoma [27]. These observations fit well with the autocrine function of activin as an inhibitory growth factor in prostate cancer cell growth, suggesting that these identified genes may play a role in the prevention of PIN to prostate carcinoma.

In contrast, the down-regulated expression of CDC6, ROK1, and hsp70 points out a balance of up- and down-regulated activin-associated genes. Interestingly, cell division cycle 6 (CDC6), a critical regulatory gene for the onset of DNA replication in eukaryotic cells, has been found to be a gene down-regulated by genistein, a soy isoflavone that inhibited prostate cancer cell growth [28], suggesting an altered interaction of transcription factors and the regulation of Cdc6. Whether hPSE interacts with CDC6 remains to be determined. Heat-shock protein70 (Hsp70) may play a role in androgen receptor synthesis and its rate of degradation and has been used to predict the outcome of prostate cancer [29]. ROK1, a putative member of the DEAD protein family of ATP-dependent RNA helicase, is essential for viability, and deletion of Rok1p inhibits pre-rRNA processing [30]. This is consistent with the activin-mediated inhibition of cell growth in prostate cancer PIN cells. We conclude that the increased ratio of up- and down-regulated activin-mediated genes may have an important role in prostate cancer progression and metastasis, reflecting in the converting of PIN to prostate carcinoma. To sum, we characterize for the first time stage-specific differentially expressed genes in PIN cells, using the gene profile from a novel approach combining *in situ* hy-

bridization, laser-capture microdissection, RNA-PCR, subtractive hybridization, and microarray technologies. A number of interesting new genes, previously not described in prostate cancer, are presented as possibilities for conversion of PIN to prostate carcinoma for further study.

Acknowledgment

This was funded by NIH/NCI Grant CA-85722.

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established that cell-specific, full-length cDNA libraries from prostate cells can be generated from small number of microdissected cells [11] and genes differentially expressed in prostate cancer have been identified using subtractive hybridization [12]. In this report, we demonstrated that several differentially expressed genes that may be associated with the regulatory conversion from PIN to prostate carcinoma, confirmed by Northern blot and microarray analyses.

Materials and method

Cell culture and treatments. The human prostatic cancer LNCaP cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD) and grown in RPMI 1640 medium supplemented with 10% fetal bovine serum with 100 µg/ml gentamicin at 37 °C under 10% CO₂. The LNCaP was passaged at ~80% confluence by exposing cells to trypsin-EDTA solution for 1 min and rinsing once with RPMI. Detached cells were replated at 1:10 dilution in fresh growth medium. After a 24-h incubation period, RNAs from the cells were isolated by either RNA-PCR or RNeasy spin column chromatography (Qiagen, Valencia, CA), fractionated on a 1% formaldehyde-agarose gel, and transferred onto nylon membranes.

RNA library amplification. The following mRNA amplification protocol is based on a published RNA-polymerase cycling reaction (RNA-PCR) procedure with minor modifications [11]. Prostatic cancer tissue sections were prefixed by 100% ethanol and dissected into homogeneous cells under a microscopic laser-capture machine (LCM; Leica, Germany). About 50 LCM-dissected cells in vivo (containing ~0.2 ng total RNAs) were ruptured by mixture with 20 µl of ice-cold cell lysis II buffer (Ambion, Austin, TX) and incubation at 75 °C for 10 min. Next the crude cell lysate was treated with DNase I (0.04 U/µl) at 37 °C for 5 min and then 75 °C for 5 min. A quarter of the crude cell lysate was applied to a reverse transcription (RT) reaction (20 µl) on ice, comprising 4 µl of 5× RT buffer (250 mM Tris-HCl, pH 8.3 at 25 °C, 375 mM KCl, 15 mM MgCl₂, 100 mM DTT), 1 µM poly(dT)₂₄ primers, dNTPs (0.5 mM each for dATP, dGTP, dCTP, and dTTP), and RNase inhibitors (40 U). After Moloney murine leukemia virus (MMLV) reverse transcriptase (100 U; Invitrogen, Carlsbad, CA) was added, the reaction was incubated at 42 °C for 1 h and shifted to 65 °C for 10 min. The function of MMLV reverse transcriptase is to not only synthesize the first strand of cDNA but also generate a short poly(dC)-tail in the 3'-end of the first-strand cDNA for RNA promoter incorporation. The first-strand cDNA–RNA hybrid so obtained was then denatured at 94 °C for 3 min and instantly mixed with 1 µM oligo(dG)-V-T7 promoter primer (5'-dephosphorylated AACGGCC AGTGAATTGTAATCGACTCACTATAAGGGAGGCGGGGGV-3', V=dA, dT or dC) at 37 °C for 1 min. A half (10 µl) of the denatured first-strand cDNA was added into a double-stranding (DS) reaction (40 µl) on ice, containing 4 µl of 10× DS buffer (500 mM Tris-HCl, pH 9.2 at 25 °C, 160 mM (NH₄)₂SO₄, and 27.5 mM MgCl₂), dNTPs (0.5 mM each for dATP, dCTP, and dTTP), and *Taq*/Pwo DNA polymerase mixture (total 5 U). The reaction was aimed to form promoter-linked double-stranded cDNA amplicon at 37 °C for 5 min and then 68 °C for 25 min. An in vitro transcription (IVT) reaction (40 µl) was then prepared for RNA amplification, containing 15 µl of the promoter-linked double-stranded cDNA amplicon, 4 µl of 10× IVT buffer (400 mM Tris-HCl, pH 8.0, at 25 °C, 60 mM MgCl₂, 100 mM DTT, and 20 mM spermidine), rNTPs (2 mM each for ATP, GTP, CTP, and UTP), and T7 RNA polymerase (200 U; Epicentre, Madison, WI). After two-hour incubation at 37 °C, the resulting RNA transcript was purified by RNA affinity resin (i.e., RNeasy spin columns, Qiagen) and directly used for another round of amplification through cycling steps of the above RT, DS, and IVT. The quality of

second-amplified RNA library (2 µg) was assessed on a 1% formaldehyde-agarose gel.

Subtraction hybridization. The following subtraction hybridization protocol is based on a published procedure with minor modifications [10,12]. The subtraction force is provided by the homologous affinity of 20 µg RNA-PCR-derived poly(A)⁺ RNA, or at least 200 µg phenol-chloroform extracted total RNAs as a subtracter library to a compared first-strand cDNA library (tester). Poly(A)⁺ RNA is selectively bound by beads (Qiagen) through poly(dT) ligand, which contains about twenty to thirty deoxythymidylate oligonucleotides. The tester cDNA library is made by Superscript-II reverse transcriptase activity (20 U) with poly(dT)₂₄ primer (1 µM) and then degrading the complementary RNA by 0.5 N NaOH for 5 min. Stored in 10 mM Tris buffer (pH 7.0), the tester cDNAs so obtained are all flanked with 3'-poly(dC) tails and 5'-poly(dT) ends. The hybridization between subtracter RNAs and tester DNAs was performed in subtractive hybridization buffer (3 mM EPPS, pH 8.0, 0.5 M NaCl, and 3 mM EDTA) at 65 °C for 30 min and occasionally mixing the reaction every 3 min for a more completely subtractive coverage. The unbound cDNAs were double-stranded by a long-template PCR system (Roche) with oligo(dG)-T7 RNA promoter primer (1 µM) at 50 °C for 5 min and then at 68 °C for 10 min. Since these double-stranded unbound cDNAs possess T7 RNA promoter in the 5'-end sense orientation, a subtracted poly(A)⁺ RNA library can be generated and re-amplified by an IVT reaction as aforementioned. The subtracted poly(A)⁺ RNA library was directly used for the following microarray analysis to show the identity of individual differentially expressed known genes under the tested comparison condition.

Microarray analysis. To prepare labeled probes for microarray hybridization, RNA (2 µg) was converted into double-stranded cDNA with a modified oligo(dT)₂₄-T7 promoter primer, 5'-GGCCAGTGGAA TTGTAATACGACTCACTATAAGGGAGGCGGGGGV-3', and the Superscript Choice system for cDNA synthesis (Gibco-BRL, Gaithersburg, MD). Double-stranded cDNA was purified by phenol-chloroform extractions, precipitated with ethanol, and resuspended at a concentration of 0.5 µg/µl in diethyl pyrocarbonate (DEPC)-treated ddH₂O. Phase-Lock Gel (5' → 3', Boulder, CO) was used for all organic extractions to increase recovery. In vitro transcription was performed with T7 RNA polymerase and with 1 µg cDNA, 7.5 mM unlabeled ATP and GTP, 5 mM unlabeled UTP and CTP, and 2 mM biotin-labeled CTP and UTP (biotin-11-CTP, biotin-16-UTP, Enzo Diagnostics). Reactions were carried out for 4 h at 37 °C and cRNA was purified by RNeasy spin columns (Qiagen). A sample was separated on a 1% agarose gel to check the size range and then 10 µg of cRNA was fragmented randomly to an average size of 50 bases by heating at 94 °C for 35 min in 40 mM Tris-acetate, pH 8.0, 100 mM KOAc/30 mM MgOAc.

A set of four oligonucleotide microarrays (human GeneChip U95A2 arrays, Affymetrix, Santa Clara, CA) containing total 12,258 genes was used for hybridization. Hybridizations were completed in 200 µl AFFY buffer (Affymetrix) at 40 °C for 16 h with constant mixing. After hybridization, arrays were rinsed three times with 200 µl of 6× SSPE-T buffer (1× 0.25 M sodium chloride/15 mM sodium phosphate, pH 7.6/1 mM EDTA/0.005% Triton) and then washed with 200 µl of 6× SSPE-T for 1 h at 50 °C. The arrays were rinsed twice with 0.5× SSPE-T and washed with 0.5× SSPE-T at 50 °C for 15 min. Staining was done with 2 µg/ml streptavidin–phycoerythrin (Molecular Probes) and 1 mg/ml acetylated BSA (Sigma) in 6× SSPE-T (pH 7.6). The arrays were read at 7.5 µm with a confocal scanner (Molecular Dynamics) and analyzed with Affymetrix Microarray Suite version 4.0 software. The samples were normalized by using the total average difference between perfectly matched probes and mismatched probes. The differential signals that were induced greater than 4-fold are collected. The 4-fold was arbitrarily chosen as the cut-off and was more conservative than the cut-off (2-fold) recommended by Affymetrix.

Northern blot analysis. RNAs (10 µg) were fractionated by electrophoresis in 1% formaldehyde-agarose gel and transferred to nylon

membranes. Synthetic 75-bp oligonucleotide probes complementary to target genes were random hexamer labeled with the Prime-It II kit (Stratagene, La Jolla, CA) in the presence of [³²P]dATP (>3000 Ci/mM, Amersham International, Arlington Heights, IL), and purified with 30 bp-cutoff Micro Bio-Spin chromatography columns (Bio-Rad, Hercules, CA). Hybridization was carried out in a mixture of 50% freshly deionized formamide (pH 7.0), 5× Denhardt's solution, 0.5% SDS, 4× SSPE, and 250 µg/µl denatured salmon sperm DNAs (18 h, 42 °C). Membranes were sequentially washed twice in 2× SSC, 0.1% SDS (10 min, 25 °C), and once in 0.1× SSC, 0.1% SDS (30 min, 42 °C) before autoradiography.

Results and discussion

About 50 high-grade PIN cells, which express activin A as determined by *in situ* hybridization, were collected by laser-capture microdissection and mRNA libraries were generated from these cell-stage specific cells using the RNA-PCR. mRNA libraries of adjacent activin-negative PIN cells were similarly prepared. Subsequently, subtractive hybridization of these two types of mRNA libraries was performed. The subtractive mRNA libraries were subjected to microarray identification (Fig. 1A). The differentially expressed genes so obtained showed a 36% degree of deviation from the results obtained without subtractive hybridization, probably resulting from the deviation of high G–C rich genes and noisy heterogeneous background before subtraction (Fig. 1B). We further demonstrated that this approach resolved the problem of heterogeneity of prostate cancer samples by comparing total RNAs conventionally extracted from large bulk of tissues with RNA-PCR-generated mRNA libraries from a small number of microdissected cells (Fig. 1C). The advantages of this approach to elucidate the differentially expressed genes rely on its ability to reveal genes usually and frequently masked in total RNAs generated from large heterogeneous prostate cancer samples. For example, the activin-induced hPSE and activin-suppressed bcl-2 expression was identified by this approach, but was not detected when bulk tissues were used for RNA extractions.

Collection of single cells from histological slides of human tissues for gene analysis was firstly reported by Becker et al. [13]. Subsequently, Schutze et al. [14] reported identification of point mutated genes by RT-PCR in isolated single cells from archived colon adenocarcinoma. Lin et al. [11] and Huang et al. [15] combined this technique with a novel method of mRNA library amplification (RNA-PCR) and identified differentially expressed genes in prostate cancer, alleviating the problem of heterogeneity of prostate cancer tissue samples. Similar advantages were observed by others [16–19]. Furthermore, this approach has been used in a new protein array technology to study specific molecular pathways [20]. To the best of our knowledge, this is the first report combining *in situ* hybridization, laser-cap-

ture microdissection, mRNA amplification, subtractive hybridization, and microarray technologies to pinpoint the differentially expressed genes in stage-specific prostate cancer cells.

In order to ascertain that the RNA-PCR-derived mRNA libraries from as few as 50 cells and total RNAs of bulk of cells extracted by phenol–chloroform were compatible, experiments were carried out, using microarray analyses, and correlation coefficient (CC) was observed between these two preparations. As shown in Fig. 2 (bottom), the RNA-PCR-amplified mRNA from 0.1 ng reference RNA (Stratagene, San Diego, CA) displayed an average of more than 98% correlation coefficient (CC) as compared to 10 µg of unamplified source reference RNAs, using Affymetrix U95A2 microarray analysis ($N = 4$). We further compared total RNA libraries extracted by RNeasy columns (Qiagen) from ~ one million human prostate cancer LNCaP cells to RNA-PCR-amplified RNA libraries from 0.1 ng of the extracted RNAs and showed a >96% correlation coefficient rate. With a pre-set threshold for acceptable variation of onefold change, such high CC rate indicates that >96% of the original mRNA population has been well preserved in almost the same composition. The often used aRNA amplification products from 0.1 ng total RNA displayed a lower 75–77% CC rate, which might have resulted from the use of random primers during cycling amplification of the aRNA amplification procedure. Other RT-PCR products from 0.1 ng total RNA usually showed a CC rate less than 75% [21]. We conclude from these results that the RNA-PCR provides a more consistent method of generating mRNA libraries, at single-cell scales, to circumvent the heterogeneity of prostate cancer samples with high fidelity, lineage, and reproducibility.

Subsequently, numerous cancer-stage-specific and cell-type-specific gene targets were identified which had been confirmed by Northern blot analysis (Fig. 3). A total of identified 42 and 72 up- and down-regulated genes were identified in activin-positive vs. activin-negative PIN cells, respectively, with 29 and 56 newly up- and down-regulated genes, respectively, when compared with previous results [10]. As indicated before, differentially expressed genes were identified previously between activin-associated PIN cells [10]. However, only 60% identified differential genes (8/13) were similar to those previously reported known genes obtained by subtractive hybridization and PCR. The remaining 40% genes (5/13) identified were only identified by the combination of subtractive hybridization and microarray technology, but were not detected by the subtractive hybridization and PCR protocol. This is probably due to the low resolution of gel electrophoresis and high noisy background of PCR amplification. We conclude that the combination of subtractive hybridization and microarray analysis offers not only a convenient tool for

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